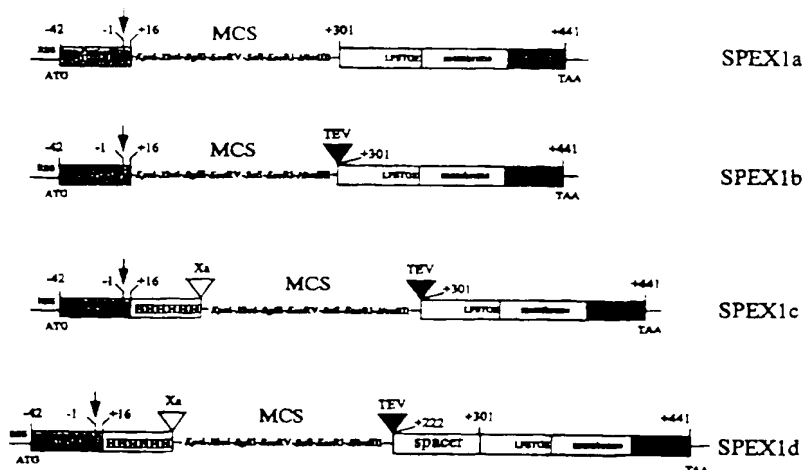




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(54) Title: USE OF GRAM-POSITIVE BACTERIA TO EXPRESS RECOMBINANT PROTEINS



(57) Abstract

A novel system for cloning and expression of genes in gram-positive bacteria. The expression system is based on the finding that many gram-positive bacteria sort proteins to their cell surface through *cis*-acting N-terminal signal sequences and C-terminal anchor regions. In particular, the cell sorting signals of the streptococcal M6 protein, a well-known surface molecule, are used to construct a gram-positive expression system, designated SPEX (Streptococcal Protein Expression). Expression is achieved by cloning the gene of interest into an appropriate SPEX cassette which is then stably introduced into a bacterial host, such as the human commensal *Streptococcus gordonii*. Depending on the SPEX vector used, recombinant proteins can be anchored to the cell wall prior to release by specific endoproteolytic cleavage or secreted into the culture medium during bacterial growth. The use of host bacteria lacking extracellular proteases should protect secreted proteins from proteolytic degradation. Several expression vectors in this system also produce specifically-tagged recombinant proteins which allows for a one-step purification of the resulting product.

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USE OF GRAM-POSITIVE BACTERIA TO EXPRESS RECOMBINANT PROTEINS

BACKGROUND OF THE INVENTION

1. *Field of the Invention*

5 The present invention relates to the construction and use of a novel gram-positive expression vector system. Protein fusions containing the amino and carboxy sorting sequences of a gram-positive surface polypeptide can be anchored to the surface of a heterologous host. Alternatively, recombinant proteins may be secreted into the growth medium. This system can be used for
10 overproducing and purifying recombinant proteins or peptides for any purpose, including but not limited to commercial scale production of diagnostic and vaccine antigens and therapeutic proteins, such as hormones and growth factors.

2. *Description of the Related Art*

 The ability to overproduce many prokaryotic and eukaryotic proteins has
15 been made possible through the use of recombinant DNA technology. The introduction of chimeric DNA molecules into *Escherichia coli* has been the method of choice to express a variety of gene products. The main impetus behind the use of *E. coli*-based protein production systems is the host's short generation time and well-developed genetics. Yet despite the development of
20 many efficient *E. coli*-based gene expression systems in recent years, the most important concern continues to be that associated with downstream processing of the product. Recombinant proteins produced in *E. coli* do not readily cross the outer cell membrane (OM); as a result, polypeptides must be purified from the cytoplasm or periplasmic space (PS). Purification of proteins from these cellular
25 compartments can be somewhat difficult. Frequently encountered problems include low product yields, contamination with potentially toxic cellular material (i.e., endotoxin) and the formation of large amounts of partially folded

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polypeptide chains in non-active aggregates, called inclusion bodies. As a result of these inherent purification difficulties, a great deal of attention has recently been focused on the natural ability of many gram-positive bacteria to export proteins beyond their cell wall boundaries. In spite of the fact that the genetics of gram-positive bacteria are not as well-elucidated as those of *E. coli*, these microorganisms have, nevertheless, become recognized as more favorable candidates as hosts for the production of recombinant proteins.

Proteins exported across the gram-positive cytoplasmic membrane (CM) generally have two fates: they are either released (secreted) into the extracellular milieu or they remain anchored to the cell wall/membrane. Many of the recently developed gram-positive based expression systems have relied exclusively on the former route of protein export (i.e., secretion). The basic strategy for directing the secretion of proteins in gram-positive expression systems involves fusing target proteins with functional N-terminal signal sequences of the gram-positive secretion systems currently available. By far, the most popular hosts belong to the genus *Bacillus*. This group of microorganisms has been used by industry for the production of a variety of economically important proteins (Priest, *Bacteriol. Rev.*, **41**:711 (1977); Glenn, *Ann. Rev. Microbiol.*, **30**:41 (1976)). Perhaps the most extensively studied gram-positive host-vector expression system is based on the *B. amyloliquefaciens* alpha-amylase gene (Ulmanen *et al.*, *J. Bacteriol.*, **162**:176 (1985); Palva *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, **79**:5582 (1982); Palva *et al.*, *Gene*, **22**:229 (1983); Lundstrom *et al.*, *Virus Res.*, **2**:69 (1985)). However, expression systems based on other gram-positive hosts such as *B. subtilis*, *B. coagulans*, *B. licheniformis* (U.S. Patents 4,824,782, 5,171,673, 4,711,843; WO patent 8,605,812; Chang, S., *Methods in Enzymology*, **153**:507-516 (1987)), *Lactococcus* and *Lactobacillus* spp. (U.S. Patent 5,242,821), *Staphylococcus* spp. (Abrahmsen *et al.*, *EMBO J.* **4**:3901-3906 (1986)), *Streptomyces* spp. (U.S. Patent 4,745,056; JP Patent 2,002,379; EP 352,707) and *Corynebacterium* spp. (U.S. Patent 4,965,197; WO Patent 9,303,158) have also been developed for general use.

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Gram-positive secretion systems provide several advantages over traditional *E. coli* based expression systems. One obvious benefit is that proteins exported beyond the cell wall usually retain their native conformation. As a result, one can take full advantage of established purification protocols which are based on the functional properties of the active protein. In addition, gram-positive expression systems usually generate higher protein yields which are generally free of potentially toxic contaminating cellular material.

There are, however, several practical limitations to the purification of extracellular proteins. The first concern is that of protein instability. Recombinant proteins secreted by many gram-positive hosts are extremely sensitive to proteolytic degradation by host-encoded extracellular proteases. The presence of such proteases can drastically affect protein yield. Fortunately, significant improvements have been achieved in maintaining the viability of secreted proteins by genetically modifying host strains with reduced extracellular protease activities (Kawamura *et al.*, *J. Bacteriol.*, **160**:442 (1984); U.S. Patent 5,084,383).

Another important point to consider when using gram-positive secretion systems is the requirement for extensive downstream processing. It is widely accepted that the purification of recombinant proteins from bulky, large volume fermentations is an extremely time consuming and costly proposition, both in terms of equipment and manpower. In those cases where the use of a gram-positive secretion system is simply not practical, it is common to resort to a less expensive, gram-negative based expression system. The decision to use *E. coli* as an alternative expression host, despite the potential pitfalls, is made simply on the basis that recombinant polypeptides remain associated with the bacterial cell (i.e., intracellular or periplasmic) and hence are generally easier to purify. Therefore, it is clear that what is needed in this art is a bacterial expression system that incorporates the salient features of both gram-positive (i.e., protein secretion) and gram-negative (i.e., protein compartmentalization) systems. A novel, but simple alternative to the currently available bacterial expression

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systems would be the development of a gram-positive system that is able to specifically anchor or attach recombinant proteins directly to the cell wall surface. In this way the anchoring process can become an integral part of the purification process. For example, cells harboring a recombinant protein
5 attached to the cell surface would be washed, collected, resuspended in a small volume and then treated with a specific agent to affect the release of the desired protein. Following removal of the bacterial cells, the resulting supernatant fluid would be highly enriched for the protein product.

The cell wall of gram-positive bacteria is a complex organelle, which is
10 assembled from peptidoglycans, carbohydrates, and proteins with different biological properties. Surface proteins differ from naturally secreted products in that the former require specific sorting signals that presumably allow them to deviate from the normal default pathway of protein export. Despite the fact that many of the biologically important gram-positive proteins, including the
15 streptococcal M protein, and the protein A and fibronectin binding proteins of *S. aureus*, are anchored at the cell surface, the cell wall of gram-positive bacteria remains a relatively unknown cellular compartment.

While the normal N-terminal export signals that serve as the basis for bacterial secretion systems have been extensively characterized (Abrahmsen *et al.*, *EMBO J.*, 4:3901-3906 (1985); Heijne and Abrahmsen, *FEBS Letters*, 244:439-446 (1989); Pugsley, *Microbio. Reviews*, 57:50-108 (1993)), the *cis*-
20 acting sorting signals that orchestrate the anchoring of cellular proteins to the gram-positive cell wall have been only recently elucidated. Sequence alignment of well over 50 gram-positive surface proteins to date (excluding those from *B. subtilis* and *S. pneumoniae*) has revealed the existence of both amino- and
25 carboxy-terminal secretion/anchoring signals. As expected, all of the proteins examined contain an N-terminal signal sequence that presumably directs them to the cellular export machinery. The C-termini of these proteins contain a predominantly hydrophobic, potential membrane spanning region that is
30 immediately followed by a charged tail (Fischetti *et al.*, *Mol. Microbiol.*, 4:1603-

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1605: Figure 1: Darkly shaded region = LP(X)TG(X) consensus sequence;
lightly shaded region = carboxy-terminal hydrophobic, potential membrane
spanning region; residues in bold = charged protein tail; Protein A =
Staphylococcus aureus protein A; M6 = *Streptococcus pyogenes* M6 protein;
5 WapA = *S. mutans* wall-associated protein A; M49 = *S. pyogenes* M49 protein;
IgA-BP = streptococcal IgA-binding protein; Protein G = streptococcal protein
G; Fn-BP = staphylococcal fibronectin-binding protein; T6 = streptococcal T6
protein; Pac = *S. mutans* surface protein; Wg2 = *S. cremoris* surface protease).
Preceding the hydrophobic domain is a proline/glycine-rich region which has
10 been predicted to span the peptidoglycan cell wall in a beta-sheet-like
conformation. Within this region, all of the surface proteins examined have a
nearly 100% conserved hexapeptide with the consensus LP(X)TG(X), where X is
usually a Thr or Ser and sometimes a Gly, Lys, or Asn (Figure 1). The
conservation of the C-terminal sequence elements (i.e., LP(X)TG(X) motif,
15 hydrophobic membrane spanning region and charged tail) suggests that the
process of sorting and anchoring polypeptides to the bacterial cell wall is shared
by many gram-positive species (Fischetti *et al.*, *Mol. Microbiol.*, **4**:1603-1605
(1990); Fischetti *et al.*, *Curr. Opinion Biotechnol.*, **4**:603-610 (1993)).

The functional importance of the carboxy-terminal sorting elements
20 identified by sequence alignment analysis has been confirmed using protein A
(Moks *et al.*, *Eur. J. Biochem.*, **156**:637-643 (1986)), a well-characterized
surface protein of *S. aureus*, as a model system. Protein A, a single polypeptide
chain, contains two major functional domains. The N-terminal domain contains
a 36 amino acid signal peptide sequence followed by several repeated sequence
25 modules (E, D, A, B, and C) that have immunoglobulin binding activity. The
C-terminal domain contains conserved sorting signals which are thought to be
responsible for anchoring protein A to the cell wall (Guss *et al.*, *Eur. J.*
Biochem., **138**:413-420 (1984); Guss *et al.*, *Eur. J. Biochem.*, **143**:685 (1984);
Fischetti *et al.*, *Mol. Microbiol.*, **4**:1603-1605 (1990)). Using deletion analysis,
30 Schneewind *et al.* (*Cell*, **70**:267-281 (1992)) confirmed that the proper sorting of

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protein A in *S. aureus* requires all three of the conserved sequence elements (i.e., -LPETGE-motif, C-terminal hydrophobic domain, and charged tail). Based on their results, Schneewind *et al.* (*Cell*, **70**:267-281 (1992)) have proposed the following sequence of events in the anchoring of protein A and presumably many other gram-positive proteins to the cell surface. First, the N-terminal signal sequence of protein A directs the polypeptide into the general secretion pathway (GSP) (for reviews, see Pugsley, *Microbio. Reviews*, **57**:50-108 (1993); Salmond and Reeves, *TIBS* **18**:7-12 (1993)). During the process of membrane translocation, the main function of the C-terminal charged tail is to prevent secretion of the protein into the medium. Translocation of protein A across the membrane presumably results in the recognition of the C-terminal sorting signal. Following recognition, the LPETGE (LPXTGX) motif is cleaved specifically between the threonine (T) and glycine (G) residues and the resulting N-terminal protein fragment is then covalently linked to the *S. aureus* cell wall (Navarre and Schneewind, *Mol. Microbiol.*, **14**:115-121 (1994)).

Because the anchor motif of gram-positive surface proteins is conserved among a wide variety of molecules and several different gram-positive species, a logical question to ask is whether it would be possible to anchor a well-known surface protein to the cell wall of a heterologous gram-positive host. Pozzi *et al.* (*Res. Microbiol.*, **143**:449-457 (1992)), using the fibrillar streptococcal M protein, were the first to address the question of protein anchoring in heterologous gram-positive hosts. Structurally, the M protein consists of an extended central alpha-helical coiled-coil rod flanked by functional end domains (Figure 2) (Phillips *et al.*, *Proc. Natl. Acad. Sci. (USA)*, **78**:4689-4693 (1981); Fischetti, *Clin. Microbiol. Reviews*, **2**:285-314 (1989))(Figure 2, (A) Structural representation of the M6 protein; (B) Linear representation of the M6 protein molecule. The map displays the N-terminal signal peptide (S), several repeated domains (A, B, C, and D), the proline/glycine rich region that contains the LPSTGE motif (LPXTGX), the C-terminal hydrophobic, potential membrane spanning domain (black bar), and charged tail (KRKEEN). Amino and carboxy

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terminal residues important for export and cell wall sorting/anchoring of the M6 protein are shaded. The anchor region is not drawn to scale.). The N-terminus contains a 42-residue signal sequence required for proper processing (Fischetti, *Clin. Microbiol. Reviews*, **2**:285-314 (1989); Hollingshead *et al.*, *J. Biol. Chem.*, **261**:1677-1686 (1986)), while the C-terminus, as determined by sequence comparisons, contains the conserved residues that are necessary for cell sorting and anchoring (Fischetti *et al.*, *Mol. Microbiol.*, **4**:1603-1605 (1990); Pancholi and Fischetti, *J. Bacteriol.*, **170**:2618-2624 (1988); Figure 1). After integration of a promoterless *emm-6.1* gene in the chromosome of the human oral commensal *S. gordonii*, it was demonstrated that the M6 protein was correctly localized to the cell wall of the heterologous host (Pozzi *et al.*, *Res. Microbiol.*, **143**:449-457 (1992)).

The next logical step was to examine the possibility of exploiting the M6 sorting signals in order to express recombinant proteins on the surface of a gram-positive host. The M6 protein has been successfully modified to deliver several heterologous antigens, such as sequences derived from the human papilloma virus type 16 E7 protein, the immunodominant epitope of HIV-1 gp120 and the major allergen of white-face hornet venom, to the surface of *S. gordonii* (Pozzi *et al.*, *Infect. Immun.*, **60**:1902-1907 (1992); Pozzi *et al.*, *Vaccine*, **12**:1071-1077 (1994); Medaglini *et al.*, *Proc. Natl. Acad. Sci. (USA)*, in press (1995)). Gram-positive oral commensal bacteria expressing recombinant fusion proteins on their cell surface have been used to elicit both a mucosal and systemic immune response to foreign antigens while colonizing the oropharynx (Fischetti *et al.*, *Curr. Opinion Biotechnol.*, **4**:603-610 (1993); Medaglini *et al.*, *Proc. Natl. Acad. Sci. (USA)*, in press (1995)).

The recent results of the M6 experiments are important for two main reasons. First, they serve to confirm the hypothesis that the mechanism of protein sorting and anchoring is an extremely well conserved, if not universal, process in gram-positive bacteria. More importantly, these experiments are evidence for proof of the concept that it is possible to exploit cell wall protein

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sorting signals for the purpose of constructing novel expression systems that anchor recombinant proteins to the surface of a gram-positive bacterial host. Therefore, in view of the aforementioned deficiencies attendant with prior art expression systems, it should be apparent that there still exists a need in the art for the construction and use of a unique gram-positive protein expression system.

SUMMARY OF THE INVENTION

Accordingly, a major objective of the present invention is to develop a gram-positive bacterial expression system that can be used for the production and purification of recombinant proteins.

It is also the objective of this invention to provide, as a specific example, the construction and use of the SPEX system (for Streptococcal protein expression), which is based on the streptococcal M6 protein. This system will allow one familiar in the art of genetic manipulation and protein biochemistry to express and purify recombinant proteins from gram-positive hosts, such as the human oral commensal *S. gordonii*.

More specifically, the present invention provides a method for expressing proteins in gram-positive bacteria, comprising selecting a heterologous bacterial host which is gram-positive, deficient in extracellular protease production, and capable of anchoring surface proteins via C-terminal sorting signals; introducing into the host a plasmid vector comprising DNA fragments encoding amino and carboxy terminal sorting sequences of a gram-positive cell wall surface protein, DNA fragments coding for the desired protein product, and transcriptional and translational control sequences that function in the chosen host; expressing a fusion protein comprising the desired protein product, an N-terminal signal sequence, and a C-terminal sorting sequence wherein the expressed protein is anchored to the host's cell surface; cleaving the anchored protein from the host's cell surface using a protease; and purifying the cleaved protein from the supernatant fluid. The present invention also provides an alternative method for expressing proteins in a gram-positive host whereby the desired protein product

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is secreted into the extracellular space, and for purifying the secreted proteins from the supernatant fluid.

Briefly, the present invention features general methods for the construction and use of a gram-positive host/vector system that uses the cell sorting signals of surface proteins for targeted attachment to the cell wall. To demonstrate the utility of this system, the invention also describes the construction of several specialized vectors, designated SPEX, which are based on the *S. pyogenes* M6 protein. These vectors are intended to be used for the expression of proteins and peptides in gram-positive bacteria such as the human oral commensal, *S. gordonii*. A variety of recombinant protein products can be expressed by selecting the appropriate SPEX cloning vector. Several protein configurations are possible such as the presence of a combination of polyhistidine tag and protein A IgG binding domains, factor Xa or TEV protease cleavage sites, and a cell wall anchoring domain LP(X)TG(X). Each of the cloning vectors acts as a shuttle plasmid which permits the initial cloning to be performed in *E. coli*, followed by transformation into a gram-positive host, such as the naturally competent *S. gordonii* V288 (Challis). Depending on the SPEX vector used, recombinant proteins can be purified from an anchored state after cleavage with a specific protease or, alternatively, from the culture medium.

With the foregoing and other objectives, advantages and features of the invention that will become hereinafter apparent, the nature of the invention may be more clearly understood by reference to the following detailed description of the preferred embodiments of the invention and to the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the alignment of the C-terminal residues from a select group of gram-positive surface proteins.

Figure 2 depicts the functional domains of the *S. pyogenes* M6 protein.

Figure 3 depicts the nucleotide sequence of the *S. pyogenes emm6* gene

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and adjacent DNA regions.

Figure 4 is a diagrammatic representation of the construction of SPEX vectors. The pBLUESCRIPT (Ap^r) backbone of each plasmid is not shown.

Figure 5 illustrates possible SPEX cassettes useful for anchored surface production and purification of recombinant proteins.

Figure 6 illustrates possible SPEX cassettes used for the secretion and purification of recombinant proteins.

Figure 7 depicts some possible *S. gordonii* strains for integrated expression of M6 fusion proteins.

Figure 8 depicts integration of the M6 fusions into the *S. gordonii* chromosome.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

More particularly, the present invention relates to general methods for the construction and use of a novel gram-positive expression system. Proteins produced by the expression system described herein can be purified either directly from the cell surface or from the supernatant fluid following secretion. For example, an expression system based on the well-known M6 protein of *S. pyogenes* is also described. This expression system, designated SPEX (for Streptococcal protein expression), uses the human oral commensal *Streptococcus gordonii* as a model host for this group of organisms.

A. General Method

The first major factor to be considered when constructing the gram-positive expression system is to determine which bacterium to use as a host. To be effective in the present invention the bacterial host should be a gram-positive bacteria that is capable of anchoring surface proteins via C-terminal sorting signals. Such gram-positive bacteria include, but are not limited to, the genera

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Aerococcus, *Bifidobacterium*, *Corprococcus*, *Deinobacter*, *Deinococcus*,
Enterococcus, *Gemella*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*,
Marinococcus, *Melissococcus*, *Micrococcus*, *Pediococcus*, *Peptococcus*,
Peptostreptococcus, *Planococcus*, *Ruminococcus*, *Saccharococcus*, *Salinococcus*,
5 *Carcina*, *Staphylococcus*, *Stomatococcus*, *Streptococcus*, *Trichococcus*, and
Vagococcus. To gain the maximum benefit from the present invention, the
bacterial host should be deficient in extracellular protease production.

In the present invention the more preferred species to be used as an
expression host is *Streptococcus gordonii*. The most preferred strain is a
10 protease deficient variant of V288 (Challis).

The second major consideration is the selection of the gram-positive cell
wall protein to be used as the basis for the construction of the expression vectors.
Nearly all surface molecules of gram-positive bacteria have amino and carboxy
terminal sorting signals that specifically target them for cell wall attachment
15 (Fischetti *et al.*, *Mol. Microbiol.*, **4**:1603-1605 (1990)). The expression system
used in the practice of the present invention can be constructed with any gram-
positive surface protein that contains the appropriate sorting signals. Such
sorting signals can be readily identified by those skilled in the art. Sources of
surface proteins include, but are not limited to, such gram-positive genera as
20 *Aerococcus*, *Bifidobacterium*, *Corprococcus*, *Deinobacter*, *Deinococcus*,
Enterococcus, *Gemella*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Marinococcus*,
Melissococcus, *Micrococcus*, *Pediococcus*, *Peptococcus*, *Peptostreptococcus*,
Planococcus, *Ruminococcus*, *Saccharococcus*, *Salinococcus*, *Carcina*,
Staphylococcus, *Stomatococcus*, *Streptococcus*, *Trichococcus*, and *Vagococcus*.

25 The more preferred protein to be used in the present invention is the type-
6 M protein of *Streptococcus pyogenes*. The structural gene for the type 6-M
protein from *S. pyogenes* has been cloned (Scott and Fischetti, *Science*, **221**:758-
760 (1983)) and the complete nucleotide sequence has been determined
(Hollingshead *et al.*, *J. Biol. Chem.* **261**:1677-1686 (1986)). This sequence is
30 shown in Figure 3. The predicted M6 amino acid residues are shown below the

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DNA sequence. The putative ribosome binding site (RBS) is boxed and the signal peptide (-42 to -1) is underlined. The mature polypeptide represents residues +1 to 441. The proline/glycine rich cell wall domain is shown as unshaded rectangles. The LPXTGX (LPSTGE) motif is shown in bold letters.

5 The adjacent lightly shaded rectangle represents the hydrophobic, membrane spanning domain and the darkly shaded rectangle represents the C-terminal charged tail. Relevant restriction sites are shown above the DNA sequence. A transcriptional terminator is shown as the inverted repeat structure denoted by the arrows.

10 The next consideration is to isolate and characterize the DNA sequence that codes for the cell wall protein in the chosen host. Methods used for the construction and screening of genomic libraries (i.e., plasmid, cosmid, phage or YAC) to isolate the appropriate protein coding sequence and techniques for determining its nucleotide sequence are known to those skilled in the art of
15 genetic engineering. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); Maniatis, Fritsch, & Sambrook, *Molecular Cloning: A Laboratory Manual* (1982) and Griffin & Griffin, *PCR Technology: Current Innovations* (1994).

The DNA fragments that encode the amino and carboxy terminal sorting
20 signals of the surface protein can be isolated using recombinant DNA techniques familiar to those skilled in the art. These fragments can be assembled on an *E. coli* cloning vector such as pUC18/19 or any other appropriate plasmid element. The N-terminal signal should include a complete leader sequence as well as a number of amino acid residues from the N-terminus of the mature
25 protein. The inclusion of these sequences will insure proper targeting to the cellular export machinery and correct processing during the actual translocation process. The C-terminal sorting (i.e., anchor) signals should include the LPXTGX motif (where X is any amino acid residue), a hydrophobic membrane spanning domain and a charged tail. A comparison of such sorting sequences is
30 shown in Figure 1. The darkly shaded sequences on the left side of the

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alignment represents the LP(X)TG(X) consensus sequence and the proteins were aligned from this sequence. The lightly shaded region on the right side of the alignment represents the carboxyl-terminal hydrophobic, potential membrane spanning region. Residues shown in bold represent the charged protein tail.

5 To facilitate the construction of heterologous fusion proteins, a small DNA fragment containing the recognition sequences for several restriction endonucleases can be placed between the DNA sequences that encode the amino and carboxy terminal sorting signals. This multiple cloning site (MCS) will facilitate the insertion of heterologous protein coding sequences and upon
10 expression will generate in-frame protein fusions that are anchored to the gram-positive cell wall surface. Alternatively, expression vectors can also be constructed that lack the C-terminal anchor signals. In this case, the protein fusions will not be targeted for anchoring but, instead, will be secreted into the extracellular space.

15 The vectors described in the present invention will allow one familiar in the art to produce recombinant proteins that are either anchored at the cell surface or secreted into the extracellular space. Regardless of the final destination of the target protein, all of the vectors contain the M6 ribosome binding site (RBS), translation initiation codon (ATG) and the 42 amino acid
20 leader sequence (-42 to -1) (Figure 3, 4 and 5). In addition, all of the vectors contain some portion of the mature N-terminus of the M6 protein. Previous studies have demonstrated that as many as 122 amino acids of the mature M protein N-terminus are also required for successful translocation. However, more recently, it has been shown that as few as 5 N-terminal amino acid residues
25 of the mature protein may be required for proper processing and translocation of a M6 fusion protein to the cell surface (Fischetti *et al.*, *Curr. Opinion Biotechnol.* 4:603-610 (1993)). The preferred number of M6 amino terminal residues to be used in the practice of the present invention is 58 and include residues -42 to +16 (Figure 3).

30 The preferred C-terminal sorting signals used in the present invention are

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also derived from the M protein of type-6 *S. pyogenes*. The vectors designed for targeted protein anchoring contain the M6 C-terminal residues 302 to 441, termination codon (TAA) and downstream sequences, which include a putative transcriptional terminator (Figure 3). Vectors that are designed for protein secretion do not contain the M6 C-terminal sorting signals.

The expression vector systems of the present invention are suitable for overproducing and purifying any desired protein. At the very least, the expression vector systems of the present invention are useful for overproducing and purifying recombinant proteins or peptides. These proteins or peptides may be used for any purpose; for example, they may be used to generate diagnostic and vaccine antigens and therapeutic proteins, such as hormones and growth factors, and the like.

To facilitate the release of anchored fusion proteins from the gram-positive cell wall, cleavage sites for various proteolytic enzymes can be engineered into the expression vectors. These unique sequences can optionally be incorporated into the vectors so that they reside either immediately downstream of the N-terminal signal peptide or immediately upstream of the C-terminal sorting signal domain. It is also possible to engineer cleavage sites at both locations. Proteases that may be suitable include, but are not limited to, the proteinase of tobacco etch virus (TEV), thrombin, enterokinase, and factor Xa.

A variably sized protein spacer or linker region that physically displaces the fusion protein away from the cell surface may also be incorporated into the expression vectors. It is possible that in the absence of these spacers the efficiency of protease cleavage may be reduced due to steric hinderance with the cell wall surface. These linker sequences can be incorporated between the protease recognition sequence and the C-terminal sorting signal domain.

Any unique protease cleavage site can be incorporated into the vectors of the present invention. Preferably, the protease cleavage site will be greater than 4 amino acid residues. The most preferred protease for use with the present invention is the TEV NIa proteinase. The TEV NIa proteinase cleaves a specific

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consensus cleavage site which spans the seven amino acid sequence E-X-V/I/L-Y-X-Q*S/G (X can be any amino acid residue; Dougherty *et al.*, *EMBO J.*, 7:1281-1287 (1988)). The preferred cleavage site for the present invention is E-N-L-Y-F-Q*G (Parks *et al.*, *Anal. Biochem.* 216:413-417 (1994)). TEV

5 cleavage sites can be engineered anywhere along the fusion construct; however, the preferred location is immediately adjacent to the proline/glycine rich region harboring the LPXTGX motif (see Figure 5: RBS = M6 ribosome binding site; ATG = initiation codon; MCS = multiple cloning site (MCS); black bar =

10 membrane spanning domain; closed triangles = engineered cleavage site for TEV (tobacco etch virus) NIa protease; open triangle = cleavage site for factor Xa (IEGR/); small vertical arrow 16 = putative cellular signal peptidase cleavage site; see SPEX1b and SPEX1c). The addition of a protein spacer region between the TEV recognition sequence and the glycine/proline-rich anchor region may improve TEV NIa proteinase efficiency. The preferred spacer for the

15 present invention is derived from the M6 protein and contains amino acid residues 222 to 301 (Figure 5; SPEX1d).

Following protease treatment, the released proteins can be purified in a variety of ways known in the art. Suitable purification methods include, but are not limited to, dialysis, ultrafiltration, zonal centrifugation, molecular-exclusion

20 chromatography, isoelectric precipitation, solvent fractionation, electrophoretic separation, thermal precipitation, ion-exchange chromatography, affinity chromatography, and the like. A single step purification using affinity tags is preferred. These affinity tags, like the protease cleavage sites, can be optionally engineered at either the amino or carboxy terminal regions of the fusion protein.

25 Useful affinity tags include, but are not limited to, a polyhistidine tract (HHHHHH), the IgG binding domain of protein A and glutathione S-transferase (GST). Recombinant proteins released from the surface of a gram-positive host can be easily purified in a one step process using metal chelation (e.g., Ni-agarose), protein A-Sepharose, and glutathione-Sepharose column

30 chromatography. N-terminal signal and affinity tag sequences can be easily

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removed by incorporating a protease cleavage site that is different from the recognition sequence used to remove the protein from the cell surface.

The preferred affinity tag for the present invention is a consecutive stretch of 6 to 10 histidine residues (HHHHHH). A polyhistidine tag of six amino acid residues has been shown to be poorly immunogenic and rarely affects protein function and structure. The polyhistidine affinity tag can be engineered at either the amino or carboxy terminus of the protein. For the present invention, the preferred site is immediately downstream of the N-terminal M6 sequences (Figure 5).

The N-terminal M6 and affinity tag sequences can be removed from the purified proteins by engineering in a second protease cleavage site. The preferred enzyme for the present invention is factor Xa. The preferred location is immediately downstream of the affinity tag (Figure 5).

In order to efficiently express the recombinant gene which encodes the fusion protein, regulatory sequences should preferably be included that assure adequate transcription and translation. These vectors should contain transcriptional (i.e., promoters) and translational (i.e., ribosome binding site) control sequences that function in the chosen gram-positive host.

A number of promoters may optionally be used in the practice of the present invention. These regulatory sequences, which are known to those skilled in the art, include heterologous promoters from both gram-negative and gram-positive bacteria. These regulatory sequences can cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus. Alternatively, constitutively expressed promoter elements can also be used.

Gene dosage is a variable that has been shown to possibly have an effect on protein production in several bacterial expression systems. In general, genes present on multiple copy plasmids generate higher levels of protein. The plasmids used for the expression of recombinant proteins in the present invention should be capable of stably replicating in the chosen gram-positive host. These plasmids, which replicate at either a high or low copy number, are familiar to

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those skilled in the art.

In the present invention, the extrachromosomal elements used in plasmid-linked expression of recombinant genes may optionally contain appropriate genetic markers for selection purposes. Suitable genetic markers include various antibiotic and heavy metal resistance genes as well as nutritional determinants. However, other genes that provide a selective advantage can also be used. Ideally the selective markers should be capable of being expressed in both *E. coli* and gram-positive hosts. This would allow one to carry out the initial cloning in *E. coli* with subsequent transfer of the recombinant plasmid to a gram-positive host. Furthermore, in order to facilitate the insertion of heterologous DNA fragments, plasmids should also contain an in-frame polylinker or MCS which includes useful restriction endonuclease cleavage sites.

Techniques for introducing recombinant plasmids into nontransformable hosts are familiar to those skilled in the art. These methods include, but are not limited to, electroporation (Dower, in *Genetic Engineering-Principles and Methods* Vol.12, pp.275-296 (1990)), chemical transformation, conjugation, transduction, and the like. Recombinant DNAs can be easily introduced into those gram-positive species that are naturally competent by transformation.

The presence of certain genes on multiple copy plasmids can occasionally cause toxicity and, in some cases, cell death. The toxic effects of these gene products can many times be reduced by simply decreasing the copy number of the determinant to be expressed. This can be readily achieved by stably integrating the gene encoding the fusion product directly into the host chromosome. The genetic methods that are commonly used to create single copy prokaryotic expression modules involve homologous, site specific and illegitimate (transposition) recombination.

The preferred method of gene expression for the present invention involves integration of the recombinant SPEX construct into the *S. gordonii* chromosome. *S. gordonii* strains that are useful as expression hosts include GP230 (Pozzi *et al.*, *Res. Microbiol.* 143:449-457 (1992)), GP232 (Pozzi *et al.*,

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Infect. Immun., 60:1902-1907 (1992)) and GP251 (Pozzi *et al.*, *Vaccine*, 12:1071-1077 (1994); Figure 7: *emm-6* is the structural gene for the *S. pyogenes* type 6 M protein. The *ermC* and *cat* genes encode erythromycin and chloramphenicol resistance, respectively. Thin lines flanking the inserted (boxed) sequences represents the *S. gordonii* chromosome. The integrated sequences are located downstream of a strong chromosomal *S. gordonii* promoter (P)). These strains contain genetic cassettes that have been integrated downstream of a strong *S. gordonii* promoter via homologous recombination. In the present invention, these cassettes provide flanking regions of homology that will allow one skilled in the art to replace the sequences present on the GP230, GP232 and GP251 with the recombinant SPEX plasmids constructed *in vivo*.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE 1: Construction of SPEX plasmids

A. Anchored vectors

Plasmid pVMB20 (Pozzi *et al.*, *Infect. Immun.* 60:1902-1907 (1992)) was constructed by subcloning a 3.4 kb *ClaI* fragment, which contains a promoterless *emm-6* gene and a gene encoding erythromycin resistance (*ermC*; Horinouchi and Weisblum., *J. Bacteriol.* 150:804-814 (1982)), from pVMB3 (Pozzi *et al.*, *Res. Microbiol.* 143:449-457 (1992)) into the plasmid pBLUESCRIPT. A 545 bp interior region of the *emm-6* gene was removed by *KpnI* and *HinDIII* digestion and replaced with a small DNA fragment harboring a synthetic multiple cloning site (MCS). The resulting plasmid, designated pSMB55 (Figure 4), contains sequences encoding the first 122 N-terminal and the last 140 C-terminal amino acids of the M6 protein. DNA fragments encoding heterologous protein sequences are inserted into the MCS of pSPEX1a or pSPEX2a to form in-frame fusions with the M6 N-terminal and/or C-terminal sequences.

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The *SacI*-*HpaI* fragment from within the *ermC* determinant of pSMB55 was removed and replaced with a fragment harboring the *aphIII* gene encoding kanamycin resistance (Trieu-Cuot and Courvalin, *Gene*, 23:331-341 (1983)). The resulting plasmid was designated pSMB113 (Figure 4).

5 Plasmid pSMB104 carries the *emm-6*_{Δ104} gene encoding for M6_{Δ104}, a protein containing the first 16 N-terminal and the last 220 C-terminal amino acids (Pozzi, G., unpublished data). The *ClaI*-*KpnI* fragment of pSMB104 was used to replace the existing *ClaI*-*KpnI* fragment of pSMB113. The resulting plasmid vector, designated pSPEX1a (Figure 4), can be used to construct protein fusions
10 that contain the N-terminal 58 and C-terminal 140 amino acids of the M6 protein. These protein fusions, when expressed in an appropriate gram-positive host, will be anchored to the bacterial cell surface (Figure 5).

B. Secretion vectors

Deletion of the *HinDIII*-*SacI* fragment of pSPEX1a effectively removes
15 the C-terminal sorting signals of the M6 protein. The resulting plasmid, designated pSPEX2a, retains the M6 N-terminal 58 amino acid residues necessary for protein translocation and processing (Figure 4). As a result, protein fusions generated with pSPEX2a will be secreted into the growth medium. TEV proteinase cleavage sites and polyhistidine tags can be
20 incorporated to facilitate the removal of non-essential protein sequences and for purification purposes, respectively (Figure 6: RBS = M6 ribosome binding site; ATG = initiation codon; Term = termination codon; triangle = engineered cleavage site for TEV (tobacco etch virus) NIa proteinase; shaded residues = important for proper processing and secretion of M6; small vertical arrow =
25 putative cellular signal peptidase cleavage site).

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EXAMPLE 2. Expression of recombinant genes in *S. gordonii*

In the present invention, recombinant genes encoding M6 fusion proteins can be expressed from multicopy plasmids. Since the preferred expression host in the present invention is *S. gordonii*, plasmids containing an origin of replication (*oriV*) that functions in a variety of streptococcal species can be used
5 replication (*oriV*) that functions in a variety of streptococcal species can be used (Horodniceanu *et al.*, *Antimicrob. Agents Chemother.*, **10**:795-801 (1976); Clewell *et al.*, *J. Bacteriol.*, **117**:283-289 (1974); Behnke and Ferretti, *Plasmid*, **4**:130-138 (1980); Macrina *et al.*, *Infect. Immun.* **28**:692-699 (1980); Macrina *et al.*, *Gene* **19**:345-353 (1982)). Alternatively, recombinant genes can be
10 expressed in the form of single copies which have been stably inserted into the *S. gordonii* chromosome.

The recombinant plasmid is naturally linearized during transformation (Pozzi *et al.*, *Res. Microbiol.*, **141**:659-670 (1990); Pozzi *et al.*, *Infect. Immun.*, **60**:1902-1907 (1992)) and recognition of the flanking homologous segments
15 facilitates integration of the M6 gene fusion, together with the *aphIII* gene (Figure 8: the *cat* gene present in the *S. gordonii* recipient strain GP251 confers chloramphenicol resistance). Recombinant *S. gordonii* are selected for kanamycin resistance (conferred by *aphIII*) using the "multilayer" plating technique as previously described by Pozzi *et al.* (*FEMS Microbiol. Lett.*, **48**:
20 189-194 (1987)). Kanamycin-resistant transformants are then scored for loss of either erythromycin (conferred by *ermC*) or chloramphenicol (conferred by *cat*) resistance (depending on the recipient). Antibiotics are used at the following concentrations: erythromycin, 5 µg/ml, chloramphenicol, 5 µg/ml, and
kanamycin, 500 µg/ml. Southern blot (Southern, *J. Mol. Biol.*, **98**:503 (1975);
25 Southern, *Methods Enzymol.*, **69**:152 (1980)) or PCR analysis of purified genomic DNA of Km^r(Cm^r or Em^r) transformants can be used to establish the structure and copy number of the expression cassette in the bacterial chromosome.

Production of the recombinant protein can be determined by Western blot

analysis of whole cell extracts (Towbin *et al.*, *Proc. Natl. Acad. Sci. (USA)*, **76**:4350 (1979); Towbin and Gordon, *J. Immunol.*, **72**:313 (1984)). Surface expression of the M6 fusion protein can be tested by immunofluorescence according to the procedure described previously (Pozzi *et al.*, *Vaccine*, **12**:1071-1077 (1994)). Recombinant proteins can be detected using polyclonal antisera against the entire M6 molecule, monoclonal antibodies directed against specific M6 epitopes (Jones *et al.*, *J. Exp. Med.*, **164**:1226-1238 (1986); Jones and Fischetti, *J. Exp. Med.*, **167**:1114-1123 (1988)) or antisera directed against the heterologous protein expressed as a part of the fusion.

EXAMPLE 3. Purification of recombinant proteins
from *S. gordonii*

Recombinant *S. gordonii* are grown in an appropriate liquid medium such as Todd Hewitt-Yeast Extract Broth (THYEB), brain heart infusion (BHI) or trypticase soy broth (TSB) containing kanamycin at 37°C for 24 hours or until stationary phase growth is achieved. The bacterial culture is centrifuged and depending on the location of the recombinant protein (cell-wall anchored or secreted) the cell pellet or the spent growth medium is retained for further analysis.

20 *S. gordonii* cells harboring surface expressed proteins are collected, washed and resuspended in a minimal volume of PBS (150 mM NaCl, 16 mM Na₂HPO₄, 4mM NaH₂PO₄ pH 7.3), in the presence of proteinase inhibitors.

Recombinant proteins with engineered protease cleavage sites are removed from the cell surface by the addition of TEV Nla proteinase. Following incubation for a suitable period of time at 25-30°C, the cells are removed by centrifugation and the supernatant fluid, which is enriched for the recombinant protein, is collected. Those fusion proteins containing tandem histidine residues can be further purified by passing the supernatant over a nickel-chelating resin (ProBond, Invitrogen Corp.) or nickel nitrilotriacetic acid [Ni-NTA] agarose

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(Qiagen Inc.). Following washing, the recombinant protein is then eluted from the column using an imidazole gradient under mild conditions (i.e., pH 6.0). The amount of purified protein can be measured by the method of Bradford (Bradford, *Anal. Biochem.*, **72**:248-254 (1976)).

- 5 The use of the SPEX2 series of vectors (Figure 6) generates fusion proteins that are secreted into the surrounding medium during growth. Following removal of the bacterial cells, the spent growth medium is retained and used as the starting material for purification of the desired product. Recombinant proteins can be purified using metal chelation chromatography, as
10 described above, and non-essential N-terminal residues can be removed with TEV Nla proteinase treatment.

- While the invention has been described and illustrated herein by references to various specific materials, procedures, and examples, it is understood that the invention is not restricted to the particular combinations of
15 materials and procedures selected for that purpose. Numerous variations of such details can be implied and will be appreciated by those skilled in the art.

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WHAT IS CLAIMED IS:

1. A method for expressing a desired protein in gram-positive bacteria,
comprising:
 - selecting a heterologous bacterial host which is
 - 5 gram-positive, and
 - capable of anchoring surface proteins via C-terminal sorting signals;
 - introducing into the host a plasmid vector comprising
 - DNA fragments encoding
 - amino and carboxy terminal sorting sequences of a gram-positive
 - 10 cell wall surface protein and
 - the desired protein, and
 - transcriptional and translational control sequences that function in the
 - chosen host;
 - expressing a fusion protein comprising
 - 15 the desired protein,
 - an N-terminal signal sequence, and
 - a C-terminal sorting sequence
 - wherein the expressed fusion protein is anchored to the host's cell
 - surface;
 - 20 cleaving the anchored protein from the host's cell surface using a
 - protease; and
 - recovering and purifying the cleaved protein.
2. The method of claim 1 wherein the host is selected from the genus
Aerococcus, *Bifidobacterium*, *Corprococcus*, *Deinobacter*, *Deinococcus*,
25 *Enterococcus*, *Gemella*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*,
Marinococcus, *Melissococcus*, *Micrococcus*, *Pediococcus*, *Peptococcus*,
Peptostreptococcus, *Planococcus*, *Ruminococcus*, *Saccharococcus*,
Salinococcus, *Carcina*, *Staphylococcus*, *Stomatococcus*, *Streptococcus*,
Trichococcus, or *Vagococcus*.

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3. The method of claim 1 wherein the host is from the genus *Streptococcus*.
4. The method of claim 1 wherein the host is *Streptococcus gordonii*.
5. The method of claim 1 wherein the host is *Streptococcus gordonii* V288 (Challis), GP230, GP232, or GP251.
- 5 6. The method of claim 1 wherein the host is deficient in extracellular protease production.
7. The method of claim 1 wherein the gram-positive cell wall surface protein is derived from the genus *Aerococcus*, *Bifidobacterium*, *Corprococcus*,
10 *Deinobacter*, *Deinococcus*, *Enterococcus*, *Gemella*, *Lactobacillus*,
Lactococcus, *Leuconostoc*, *Marinococcus*, *Melissococcus*, *Micrococcus*,
Pediococcus, *Peptococcus*, *Peptostreptococcus*, *Planococcus*, *Ruminococcus*,
Saccharococcus, *Salinococcus*, *Carcina*, *Staphylococcus*, *Stomatococcus*,
Streptococcus, *Trichococcus*, or *Vagococcus*.
8. The method of claim 7 wherein the gram-positive cell wall surface protein is
15 the type-6 M protein of *Streptococcus pyogenes*.
9. The method of claim 7 wherein the gram-positive cell wall surface protein comprises:
 - 20 an N-terminal signal sequence comprising a complete leader sequence and a number of amino acid residues from the N-terminus of the mature protein;
 - a C-terminal sorting signal comprising the LPXTGX motif, wherein X is any amino acid residue, residing within a proline/glycine rich region;
 - a hydrophobic membrane spanning domain; and
 - a charged tail.

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10. The method of claim 9, wherein the gram-positive cell wall surface protein additionally comprises a DNA fragment having a recognition sequence for at least one restriction endonuclease (the multiple cloning site, MCS) located between the DNA sequences that encode the amino- and carboxy-terminal sorting signals.
11. The method of claim 1 wherein the plasmid vector further comprises at least one cleavage site for a proteolytic enzyme.
12. The method of claim 1 wherein the protease is the proteinase of tobacco etch virus (TEV), thrombin, enterokinase, or factor Xa.
13. The method of claim 9 wherein the plasmid vector additionally comprises a variably sized protein spacer or linker sequence that physically displaces the fusion protein away from the cell surface.
14. The method of claim 13 wherein the linker sequence is incorporated between the protease recognition sequence and the C-terminal sorting signal domain.
15. The method of claim 1 wherein the cleaved proteins are purified in a single step through the use of affinity tags.
16. The method of claim 15 wherein the affinity tags are polyhistidine tracts containing from 6 to 10 histidine residues, the IgG binding domain of protein A, or glutathione S-transferase.
17. The method of claim 1 wherein the cleaved proteins are purified using metal chelation, protein A-Sepharose, glutathione-Sepharose column chromatography, or a combination thereof.

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18. The method of claim 1 wherein the plasmid vector additionally comprises extrachromosomal genetic markers that are sufficient to provide a selective advantage to a host expressing said genetic markers.
19. The method of claim 18 wherein the genetic markers are antibiotic genes,
5 heavy metal resistance genes, and nutritional determinants.
20. The method of claim 18 wherein the genetic markers are capable of being expressed in both *E. coli* and gram-positive hosts.
21. The method of claim 18 wherein the plasmid vector additionally comprises a
10 polylinker or multiple cloning site which includes restriction endonuclease cleavage sites.
22. The method of claim 7 wherein the plasmid vector comprises sequences coding for the M6 ribosome binding site, the translation initiation codon (ATG), the 42 amino acid leader sequence (-42 to -1), and a portion of the mature N-terminus which is at least 5 amino acids long.
- 15 23. The method of claim 22 wherein the portion of the N-terminus coded for comprises residues -42 to +16.
24. The method of claim 1 wherein the C-terminal sorting signal is derived from the M protein of type-6 *S. pyogenes*.
- 20 25. The method of claim 1 wherein the plasmid vector additionally comprises the M6 C-terminal residues 302 to 441, termination codon (TAA), and downstream sequences which comprise a transcriptional terminator.
26. The method of claim 1, wherein the plasmid vector is pSPEX1a.

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27. The method of claim 26, wherein the pSPEX1a vector additionally comprises a protease which has a cleavage site of greater than 4 amino acid residues.
28. The method of claim 26, wherein the protease is the TEV NIa proteinase, which cleaves a specific consensus cleavage site which spans the seven amino acid sequence E-X-V/I/L-Y-X-Q*S/G, wherein X can be any amino acid residue.
29. The method of claim 28, wherein the cleavage site is E-N-L-Y-F-Q*G.
30. The method of claim 28, wherein the cleavage site is located adjacent to the proline/glycine-rich region harboring the LPXTGX motif.
31. The method of claim 28, wherein a protein spacer is added between the TEV proteinase recognition sequence and the proline/glycine-rich anchor region.
32. The method of claim 31, wherein the spacer is derived from the M6 protein and consists of amino acid residues 222 to 301.
33. A method for expressing a desired protein in gram-positive bacteria, comprising:
- selecting a heterologous, gram-positive bacterial host;
 - introducing into the host a plasmid vector comprising DNA fragments encoding
 - amino and carboxy terminal sorting sequences of a gram-positive cell wall surface protein, and
 - the desired protein; and
 - transcriptional and translational control sequences that function in the chosen host;
 - expressing a fusion protein comprising

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the desired protein, and
an N-terminal signal sequence
wherein the expressed protein is secreted from the cell; and
purifying the protein product from the supernatant fluid following
secretion.

- 5
34. The method of claim 33 wherein the host is selected from the genus *Aerococcus*, *Bifidobacterium*, *Corprococcus*, *Deinobacter*, *Deinococcus*, *Enterococcus*, *Gemella*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Marinococcus*, *Melissococcus*, *Micrococcus*, *Pediococcus*, *Peptococcus*,
10 *Peptostreptococcus*, *Planococcus*, *Ruminococcus*, *Saccharococcus*, *Salinococcus*, *Carcina*, *Staphylococcus*, *Stomatococcus*, *Streptococcus*, *Trichococcus*, or *Vagococcus*.
35. The method of claim 33 wherein the host is from the genus *Streptococcus*.
36. The method of claim 33 wherein the host is *Streptococcus gordonii*.
- 15 37. The method of claim 33 wherein the host is *Streptococcus gordonii* V288 (Challis), GP230, GP232, or GP251.
38. The method of claim 33 wherein the host is deficient in extracellular protease production.
- 20 39. The method of claim 33 wherein the gram-positive cell wall surface protein is derived from the genus *Aerococcus*, *Bifidobacterium*, *Corprococcus*, *Deinobacter*, *Deinococcus*, *Enterococcus*, *Gemella*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Marinococcus*, *Melissococcus*, *Micrococcus*, *Pediococcus*, *Peptococcus*, *Peptostreptococcus*, *Planococcus*, *Ruminococcus*, *Saccharococcus*, *Salinococcus*, *Carcina*, *Staphylococcus*, *Stomatococcus*,

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Streptococcus, *Trichococcus*, or *Vagococcus*.

40. The method of claim 39 wherein the gram-positive cell wall surface protein is the type-6 M protein of *Streptococcus pyogenes*.
41. The method of claim 39 wherein the gram-positive cell wall surface protein
5 comprises:
 an N-terminal signal sequence comprising a complete leader sequence
 and a number of amino acid residues from the N-terminus of the mature
 protein;
 a C-terminal sorting signal comprising the LPXTGX motif, wherein X is
10 any amino acid residue, residing within a proline/glycine rich region;
 a hydrophobic membrane spanning domain; and
 a charged tail.
42. The method of claim 41, wherein the plasmid vector additionally comprises a
DNA fragment having a recognition sequence for at least one restriction
15 endonuclease (the multiple cloning site, MCS) located between the DNA
sequences that encode the amino- and carboxy-terminal sorting signals.
43. The method of claim 41 wherein the expression vector additionally comprises
a variably sized protein spacer or linker sequence that physically displaces
the fusion protein away from the cell surface.
- 20 44. The method of claim 43 wherein the linker sequence is incorporated between
the protease recognition sequence and the C-terminal sorting signal domain.
45. The method of claim 33 wherein the plasmid vector additionally comprises
extrachromosomal genetic markers that are sufficient to provide a selective
advantage to a host expressing said genetic markers.

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46. The method of claim 45 wherein the genetic markers are antibiotic genes, heavy metal resistance genes, and nutritional determinants.
47. The method of claim 45 wherein the genetic markers are capable of being expressed in both *E. coli* and gram-positive hosts.
- 5 48. The method of claim 45 wherein the plasmid vector additionally comprises a polylinker or multiple cloning site containing restriction endonuclease cleavage sites.
- 10 49. The method of claim 39 wherein the plasmid vector comprises sequences coding for the M6 ribosome binding site, the translation initiation codon (ATG), the 42 amino acid leader sequence (-42 to -1), and a portion of the mature N-terminus which is at least 5 amino acids long.
50. The method of claim 49 wherein the portion of the N-terminus coded for comprises residues -42 to +16.
- 15 51. The method of claim 33 wherein the vector is one of the SPEX2 series of vectors.
52. The method of claim 33 wherein recombinant proteins are purified using metal chelation chromatography.
53. The method of claim 33 wherein non-essential N-terminal residues can be removed with TEV N1a proteinase treatment.

ADANKAQA	LPETGE	ENPLIGTTVFGGLSLALGAALLAGRRREL	Protein A
PMKETKRQ	LPSTGE	TANPFFTAAALTMATAGVAAVVKRKEEN	M6
TKQKAKFV	LPSTGE	QAGLLLTTVGLVIVAVAGVYFYRTRR	WapA
AMTQQKRT	LPSTGE	TANPFFTAAATVMVSAGMLAIKRKEEN	M49
PMAQTKRQ	LPSTGE	ETTNPFFTA.....	IgA-BP
DDAKKAET	LPSTGE	GSNPFFTAAALAVMAGAGALAVASKRKED	Protein G
KPQSKKSE	LPETGG	EESTNKGMLFGGLFSILGLALIRRNKKNHKA	Fn-BP
IPNTKLGE	LPSTGS	IGTYLFFKAIGSAAMIGAIGIYIVKRRKA	T6
QPSSVQET	LPNTGV	TNNAYMPLLGIIGLVTSFSLLGIKAKKD	Pac
QLTSGKGA	LPKTGE	TTERPAFGFLGVIIVVILMGVLGIKRKQREE	Wg2

Figure 1

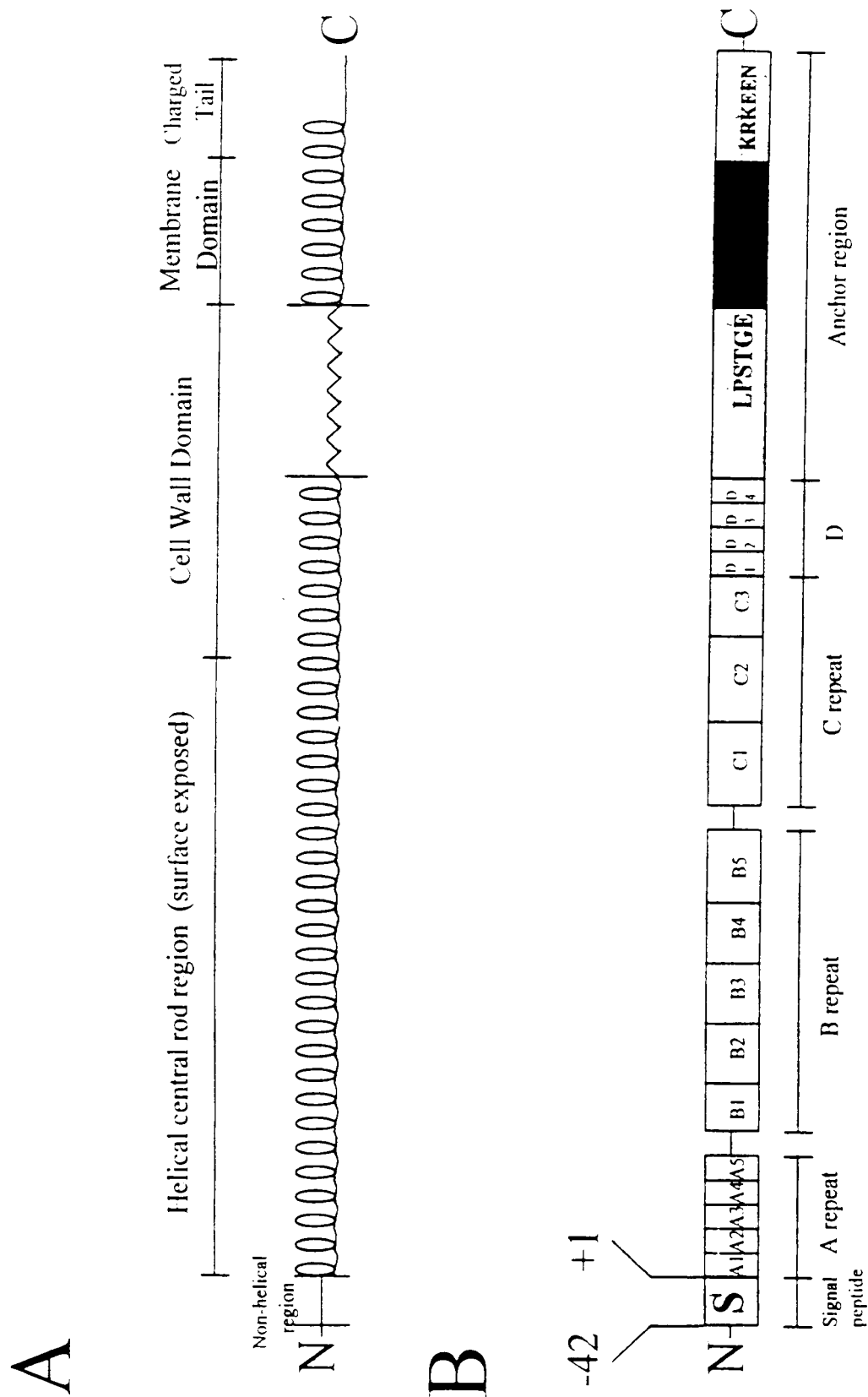


Figure 2

GTT TAC GTT TGG GGT TTT ATT ATT TAC AAT ACA ATT ATT AGA GTT AAA GCG TGA AAA TGA GGG TTT TTC GTA AAA 75
 AAT GAT AAC ATA AGG AGG ATA AAA ATG GGT AAA AAT AAC AGC AAT AGA CAC TAT TGG GTT AGA AAA TTA AAA AAA 150
 M A E N N T N E E Y E L E E L K F
 -42
 GGT ACT GCA TCA GTA GCA GTG GGT TGG AGT GTA ATA GGG GCA GGA TTA GTT GTC AAT ACT AAT GAA GTT AGT GCA 225
 Q T A S V A V A L S V I G A G L V V N T N E V S A
 -1
 AGA GTG TTT GGT AGG GGG AGC GTA GAA AAC GCG GAC AAA GCA CGA GAA GTT GTT AAC AAG TAT GAC GTA GAG AAC 300
 R V F P R G T V E N P D K A R E L L N K Y D V E N
 +1
 TGT ATG TTA CAA GGT AAT AAT GAC AAG TTA ACA ACT GAG AAT AAT AAC TTA ACA GAT CAG AAT AAA AAC TTA ACA 375
 S M L Q A N N D K L T T E N N N L T D Q N K N L T
 +16
 ACT GAG AAT AAA AAC TTA ACA GAT CAG AAT AAA AAC TTA ACA ACT GAG AAT AAA AAC TTA ACA GAT CAG AAT AAA 450
 T E N K N L T D Q N K N L T T E N K N L T D Q N K
 AAC TTA ACA ACT GAG AAT AAG GAG TTA AAA GGT GAG GAG AAT AGG TTA ACA ACT GAG AAT AAA GGG TTA ACT AAA 525
 N L T T E N K E L K A E E N R L T T E N K G L T F
 KpnI
 AAG TTG AGT GAA GGT GAA GAA GAA GCA GCA AAT AAA GAG CGA GAA AAT AAA GAA GCG ATT GGT ACC GTT AAA AAA 600
 K L S E A E E E A A N K E R E N K E A I G T L K F
 ACC TTG GAT GAG ACA GTA AAA GAT AAA ATT GGT AAG GAG CAA GAA AGT AAA GAA ACC ATT GGT ACC GTT AAA AAA 675
 T L D E T V K D K I A K E Q E S K E T I G T L F F
 ACC TTG GAT GAG ACA GTA AAA GAT AAA ATT GGT AAG GAG CAA GAA AGT AAA GAA ACC ATT GGT ACC GTT AAA AAA 750
 T L D E T V K D K I A K E Q E S K E T I G T L F F
 ACC TTG GAT GAG ACA GTA AAA GAT AAA ATT GGT AAG GAG CAA GAA AGT AAA GAA ACC ATT GGT ACC GTT AAA AAA 825
 T L D E T V K D K I A K E Q E S F E T I G T L F F
 ATC TTG GAT GAG ACA GTA AAA GAT AAA ATT GCG AGA GAG CAA AAA AGT AAA CAA GAC ATT GGT GCG GTT AAA CAA 900
 I L D E T V K D K I A R E C A K S F Q D I G A L K Q
 GAA TTA GGT AAA AAA GAT GAA GGA AAC AAA GTT TCA GAA GCA AGC CGT AAG GGT GTT CGC CGT GAC TTG GAC GCA 975
 E L A K K D E G N K V S E A S R K G L R R D L D A
 TCA CGT GAA GGT AAG AAA CAG GTT GAA AAA GAT TTA GCA AAC TTG ACT GGT GAA GTT GAT AAG GTT AAA GAA GAA 1050
 S R E A K K Q V E K D L A N L T A E L D K V K E E
 AAA CAA ATC TCA GAC GCA AGC CGT CAA GGT GTT CGC CGT GAC TTG GAC GCA TCA CGT GAA GGT AAG AAA CAA GTT 1125
 K Q I S D A S R Q G L R R D L D A S R E A K K Q V
 HindIII
 GAA AAA GGT TTA GAA GAA GCA AAC AGC AAA TTA GGT GGT GTT GAA AAA GTT AAC AAA GAG GTT GAA GAA AGC AAG 1200
 E K A L E E A N S K L A A L E K L N K E L E E S K
 AAA TTA ACA GAA AAA GAA AAA GGT GAG CTA CAA GCA AAA GTT GAA GCA GAA GCA AAA GCA CTC AAA GAA CAA TTA 1275
 K L T E K E K A E L Q A K L E A E A K A L K E Q L
 GCG AAA CAA GGT GAA GAA GTT GCA AAA CTA AGA GGT GGA AAA GCA TCA GAC TCA CAA ACC CGT GAT GCA AAA CCA 1350
 A K Q A E E L A K L R A G K A S D S V T P D A K P
 GGT AAC AAA GTT GTT CCA GGT AAA GGT CAA GCA GCA CAA GCA GGT ACA AAA GGT AAC CAA AAC AAA GCA CCA ATG 1425
 G N K V V P G K G Q A P C A G T E P N C N K A P M
 AAG GAA ACT AAG AGA CAG TTA CCA TCA AGA GGT GAA ACA GGT AAC CCA TTG TTC ACA GCG GCA GGT GTT ACT GTT 1500
 V E T K P C L P S T G E T A N P F E T A A A L T V
 ATG GCA ACA GGT GGA GTA GCA GCA GTT GTA AAA GCG AAA GAA GAA AAC TAA GGT ATC ACT TTG TAA TAC TGA GTG 1575
 M A T A G V A A V V ~~XXXXXXXXXXXX~~
 AAC ATC AAG AGA GAA CCA GTC GGT TGT CTC TTT TAT GTA TAG 1617

Figure 3

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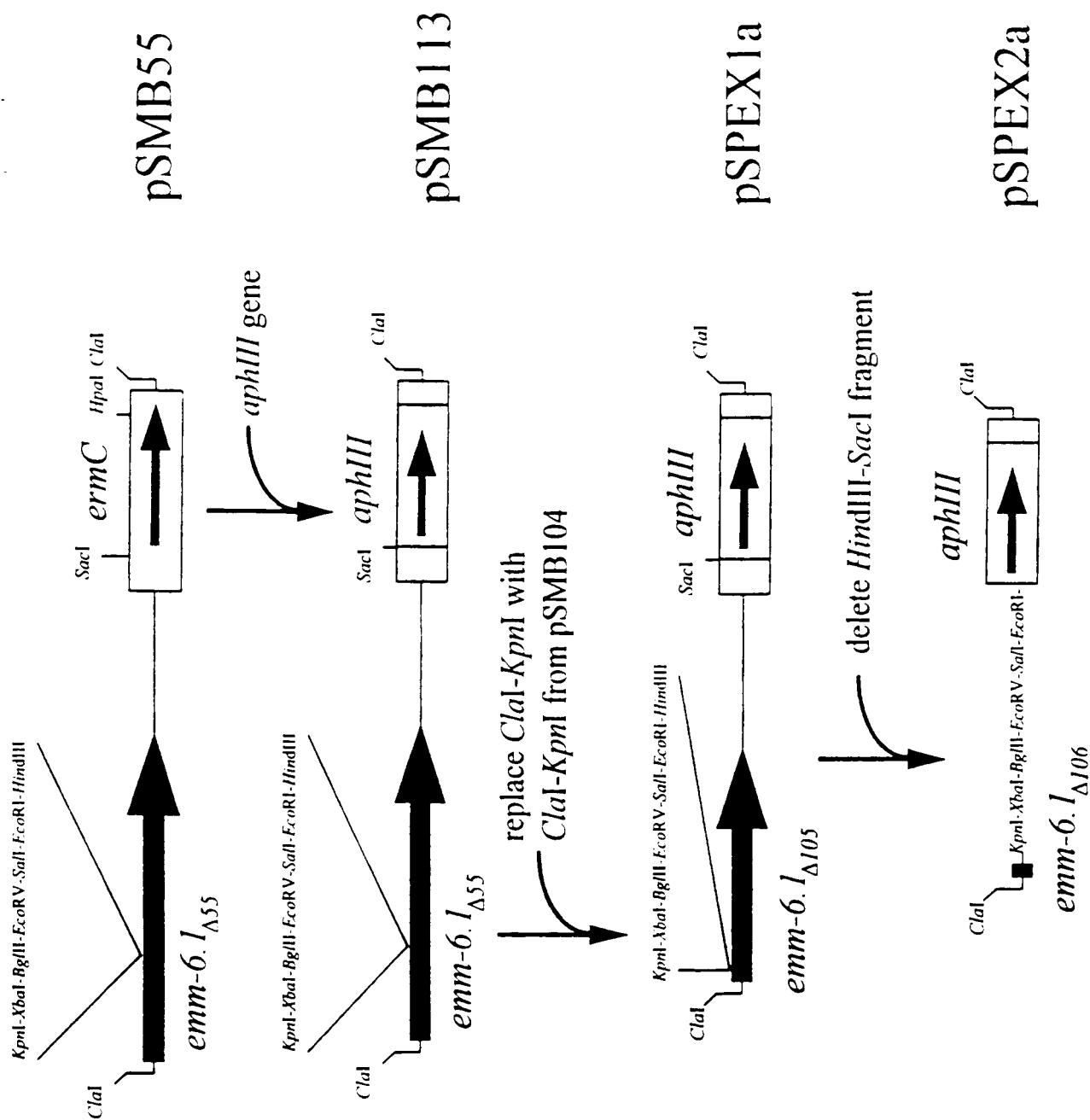


Figure 4

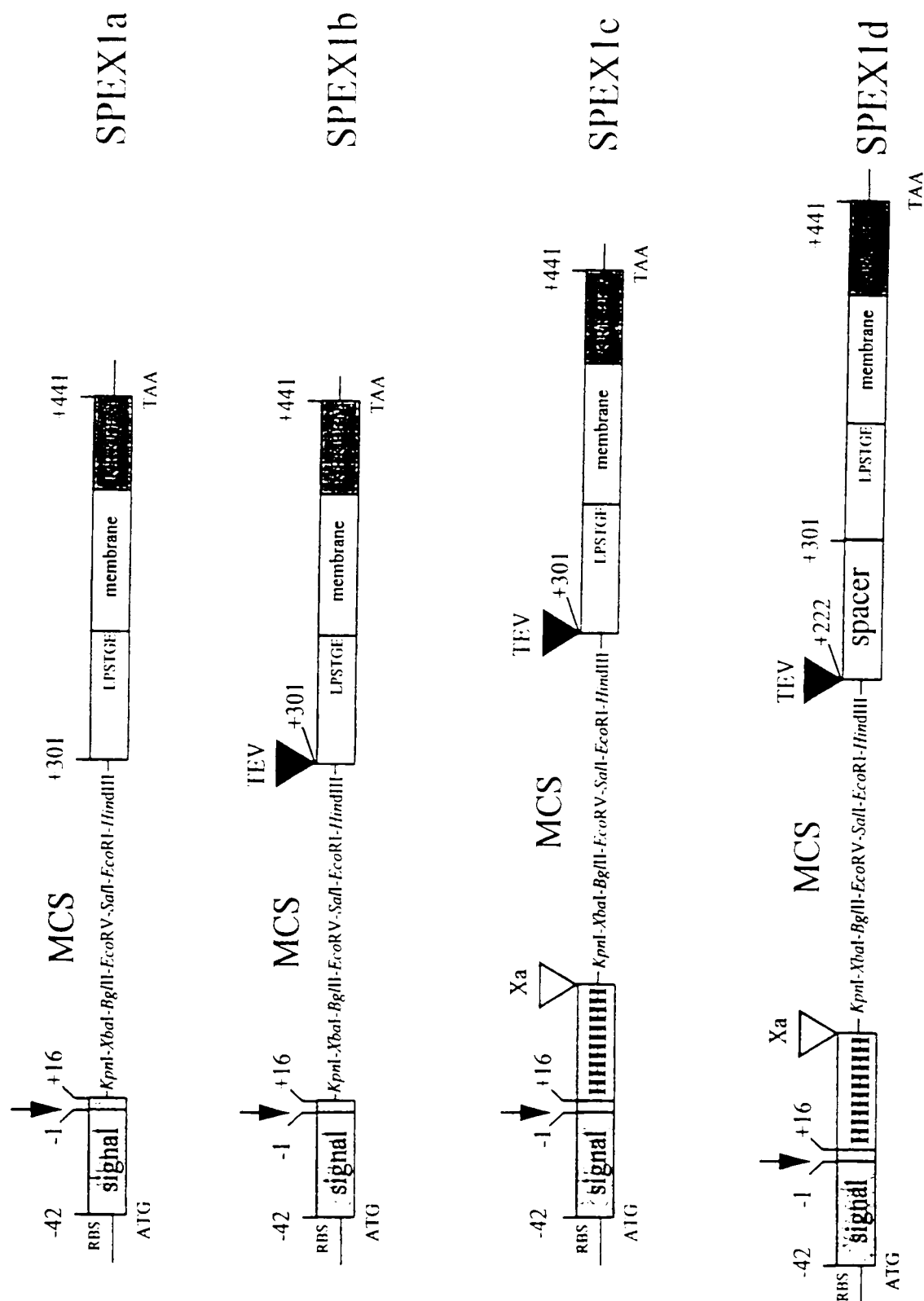


Figure 5

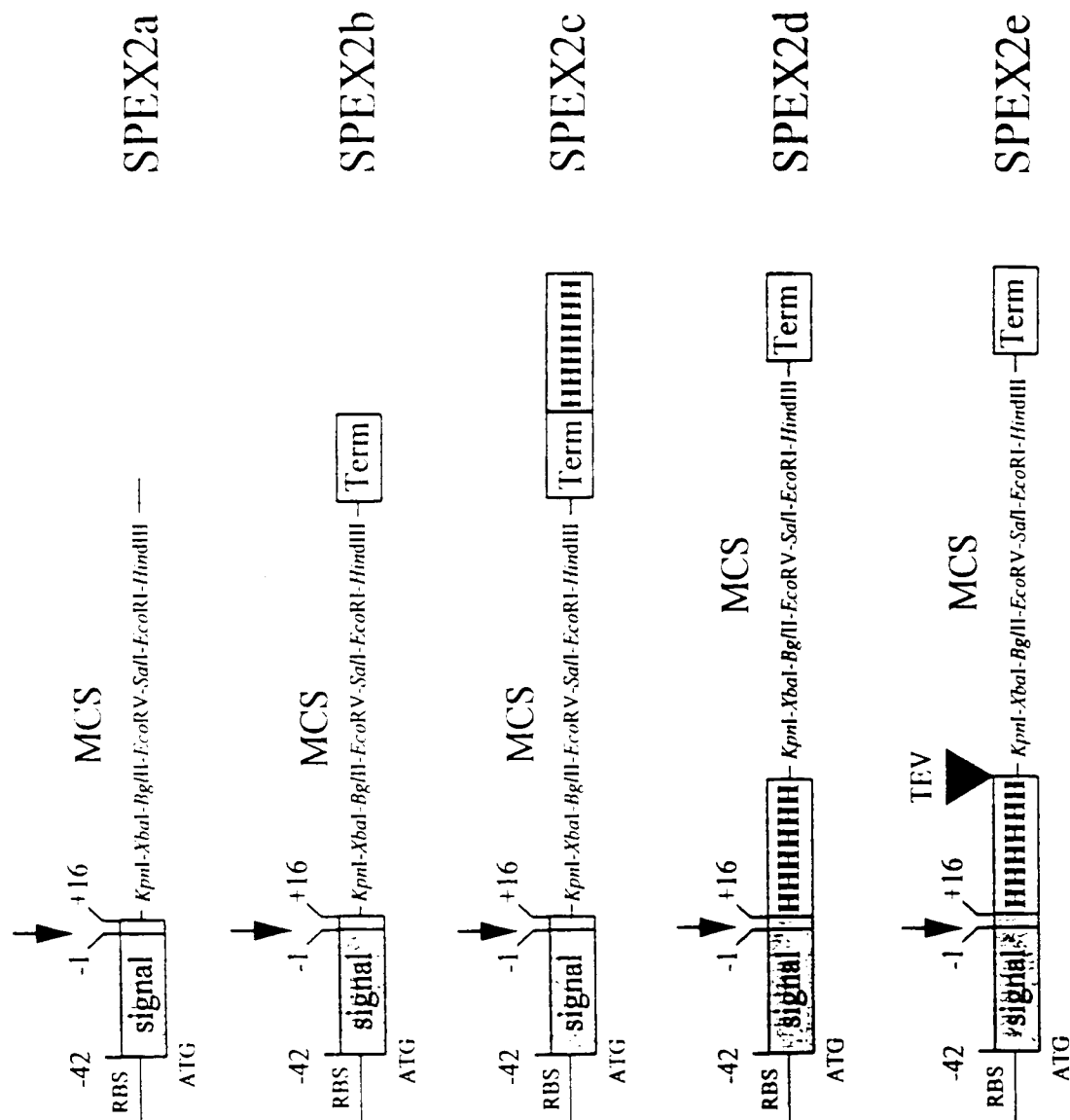


Figure 6

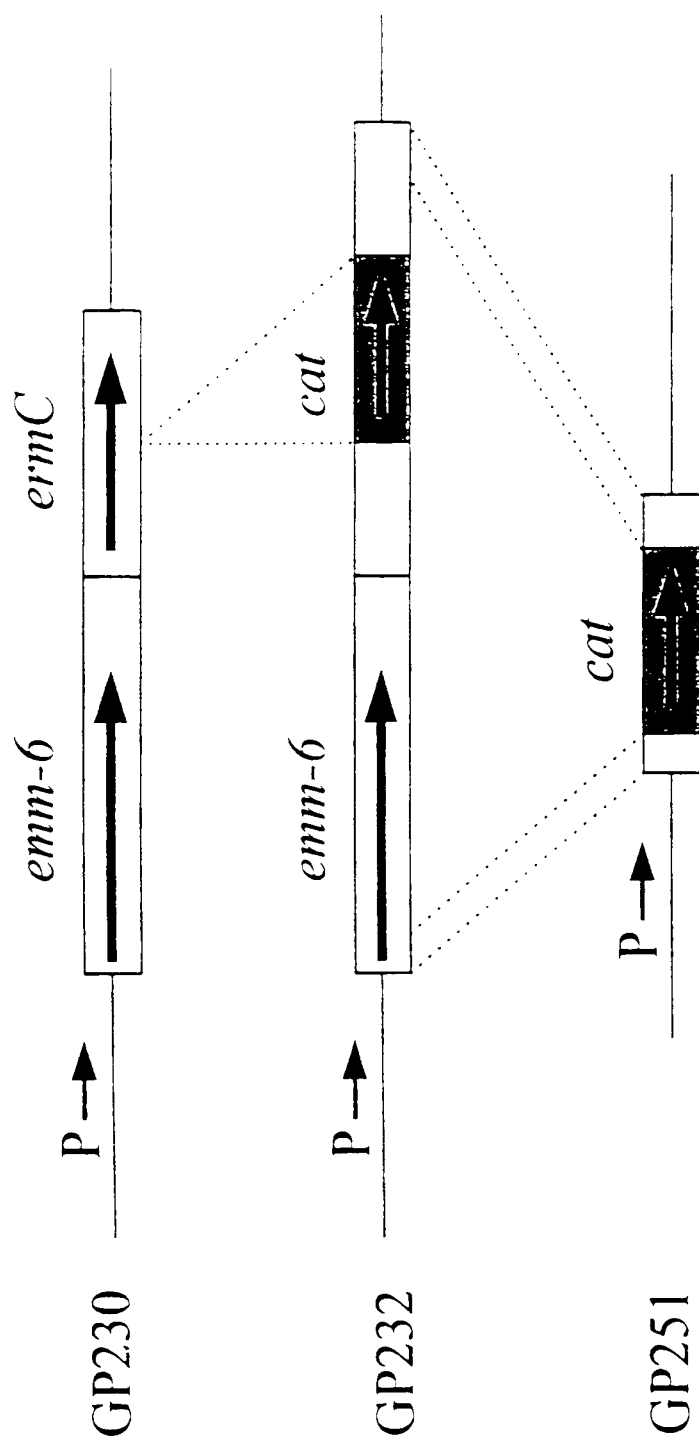


Figure 7

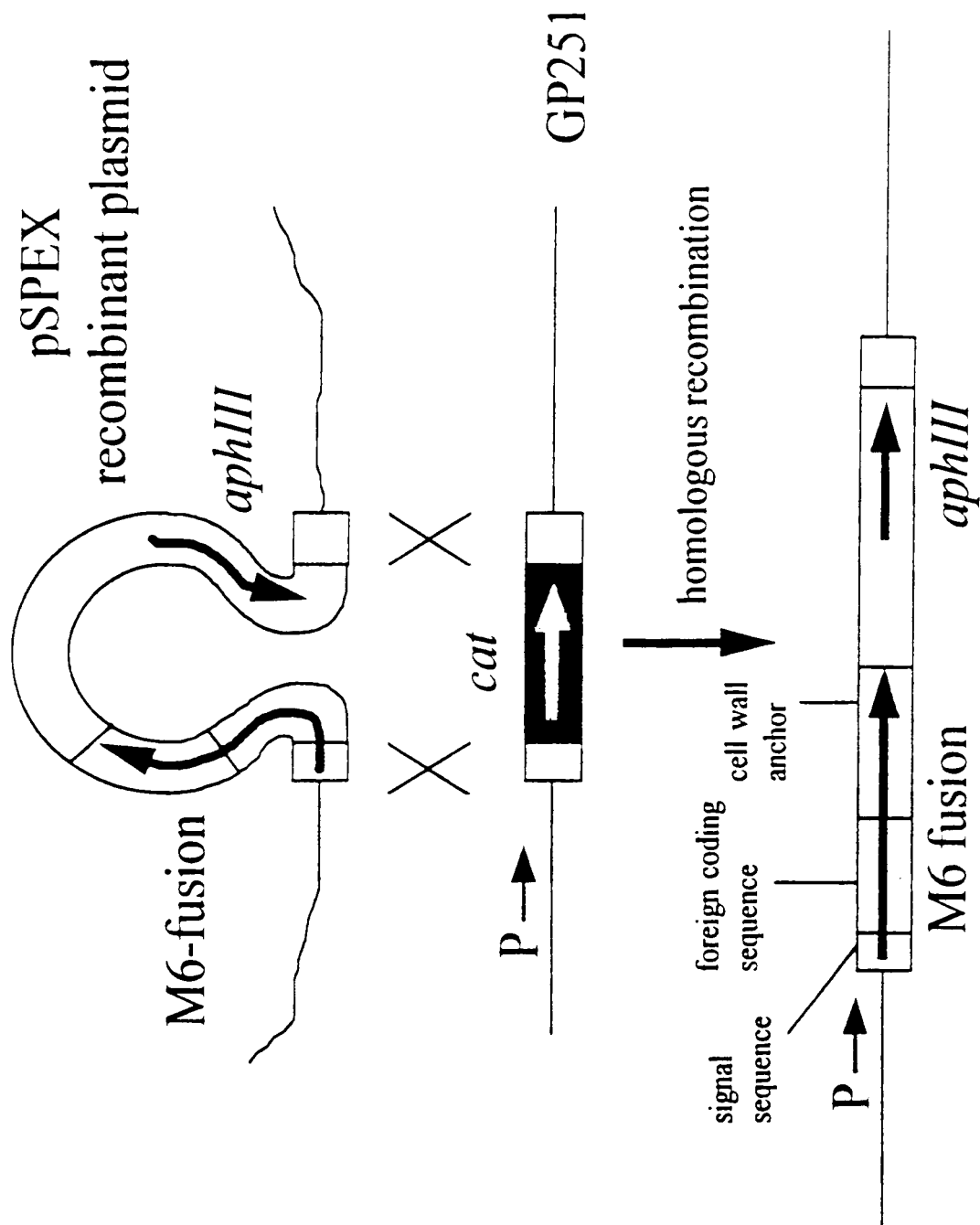


Figure 8

INTERNATIONAL SEARCH REPORT

National Application No.

PCT/US 96/09965

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/62 C12N15/31 C12N15/74 C07K14/315

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,93 18163 (UNIV ROCKEFELLER) 16 September 1993 see the whole document ---	1-5, 7-12, 18-24
Y	VACCINE, vol. 12, no. 12, 1994, BUTTERWORTH-HEINEMANN LTD, BOSTON, MA, USA, pages 1071-1077, XP002017663 G. POZZI ET AL.: "Human T-helper cell recognition of an immunodominant epitope of HIV-1 gp120 expressed on the surface of Streptococcus gordonii" cited in the application see the whole document --- -/--	1-5, 7-12, 18-24



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

5 November 1996

Date of mailing of the international search report

15. 11. 96

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Hornig, H

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 96/09965

Continuation: DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 20805 (PF MEDICAMENT) 26 November 1992 see the whole document ---	1,11,12, 18-21
Y	ANALYTICAL BIOCHEMISTRY, vol. 216, no. 1, 1 February 1994, ACADEMIC PRESS INC., DULUTH,MN,US, pages 413-417, XP002017664 T.D. PARKS ET AL.: "Release of proteins and peptides from fusion proteins using a recombinant plant virus proteinase" cited in the application see page 413, right-hand column, line 1 - line 20; figure 1 ---	1-5, 7-12, 18-24
Y	EP,A,0 355 737 (BEHRINGWERKE AG) 28 February 1990 see page 2, line 35 - line 40; claims 6,7 ---	1-5, 7-12, 18-24
Y	WO,A,93 01288 (DEUTSCHES KREBSFORSCH) 21 January 1993 see page 5, line 17 - line 33; claim 7; figure 1 ---	1-5, 7-12, 18-24
A	EMBO J., vol. 12, no. 12, December 1993, OXFORD UNIVERSITY PRESS,GB;; pages 4803-4811, XP002017665 O. SCHNEEWIND ET AL.: "Cell wall sorting signals in the surface proteins of gram-positive bacteria" see the whole document ---	1-53
A	CELL, vol. 70, 24 July 1992, CELL PRESS,CAMBRIDGE,MA,US;; pages 267-281, XP002017666 O. SCHNEEWIND ET AL.: "Sorting of protein A to the Staphylococcal cell wall" cited in the application see the whole document ---	1-53
A	EMBO J., vol. 7, no. 5, 1988, OXFORD UNIVERSITY PRESS,GB;; pages 1281-1287, XP002017667 W.G. DOUGHERTY ET AL.: "Biochemical and mutational analysis of a plant virus polyprotein cleavage site" cited in the application see the whole document -----	1-53

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/US 96/09965

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9318163	16-09-93	CA-A-	2131995	16-09-93
		EP-A-	0646176	05-04-95
		HU-A-	70306	28-09-95

WO-A-9220805	26-11-92	AU-B-	664841	07-12-95
		AU-A-	1789992	30-12-92
		CA-A-	2103021	01-12-92
		EP-A-	0584167	02-03-94
		JP-T-	7502640	23-03-95
		OA-A-	9866	15-08-94

EP-A-0355737	28-02-90	DE-A-	3828666	01-03-90
		AU-B-	614174	22-08-91
		AU-A-	4016089	01-03-90
		JP-A-	2135095	23-05-90

WO-A-9301288	21-01-93	DE-A-	4122599	04-02-93
		EP-A-	0547201	23-06-93
		JP-T-	6500930	27-01-94
